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21 environment, biocide

## Abstract

In nature and man-made environments, microorganisms reside in mixed-species biofilm where behavior is modified compared to the single-species biofilms. Pathogenic microorganisms may be protected against adverse treatments in mixed-species biofilms leading to health risk for humans. Here, we developed two mixed-five-species biofilms that included the foodborne pathogens *Listeria monocytogenes* or *Staphylococcus aureus*, respectively. The five species, including the pathogen, were isolated from a single food-processing environmental sample thus mimicking the environmental community. In mature mixed five-species biofilms on stainless steel, the two pathogens remained at a constant level of  $\sim 10^5$  CFU/cm<sup>2</sup>. The mixed-five-species biofilms as well as the pathogens in mono-species biofilms were exposed to biocides to determine any pathogen-protective effect of the mixed biofilm. Both pathogens and their associate microbial communities were reduced by peracetic acid treatments. *S. aureus* decreased 4.6 log cycles in mono-species biofilm, but the pathogen was protected in the five-species biofilm and decreased only 1.1 log cycles. Sessile cells of *L. monocytogenes* were affected equally as a mono-biofilm or as a member in the mixed-species biofilm; decreasing by three log cycles when exposed to 0.0375 % peracetic acid. When the pathogen was exchanged in each associate microbial community, *S. aureus* was eradicated while there was no significant effect of the biocide on *L. monocytogenes* or the mixed community. This indicates that particular members or associations in the community offered the protective effect. Further studies are needed to clarify the mechanisms of biocide protection, and the species playing the protective role in microbial communities of biofilms.

43 **Importance**

44 This study demonstrates that foodborne pathogens can be established in mixed species biofilms and that this  
45 can protect them from biocide action. The protection is not due to specific characteristics of the pathogen,  
46 here *S. aureus* and *L. monocytogenes*, but likely caused by specific members or associations in the mixed  
47 species biofilm. Biocide treatment and resistance is a challenge for many industries and biocide efficacy  
48 should be tested on microorganisms growing in biofilms, preferably mixed systems, mimicking the  
49 application environment.

## Introduction

To prevent contamination, infection or foodborne disease in the clinical or food producing sector, antimicrobial, detergents and biocides are used to inactivate or eradicate microorganisms (1, 2). Most guidelines for biocides include testing of efficiency on planktonic pure cultures of microorganisms but little is known about the efficacy on microbial biofilms (3). Moreover, most microorganisms live in complex biofilms (4) composed of multiple species (5, 6). In biofilms, microorganisms can cooperate (7) and protect themselves from adverse environmental conditions. Thus, several studies have reported that sessile cells can be up to 1,000 fold more resistant than cells in a planktonic state (8–10).

Biofilm formation and resistance to biocide treatment have been studied and recognized as important factors that contribute to the survival and persistence of microbial contamination in drinking water (11) and in oral hygiene (12, 13). The food processing environment is also believed to provide conditions for biofilm development including polymicrobial biofilms. Although many studies have focused on mono-culture systems, it is being recognized that biofilms are predominantly polymicrobial (14, 15). The behavior of microorganisms in a mixed biofilm differs from the behavior of a mono-species biofilm (16, 17) and for instance resistance to antimicrobials can be increased in the mixed system (18). The complete picture on mechanisms involved in resistance has not been fully unraveled, but some of the reasons for biofilm resistance to antimicrobial compounds are proposed to be caused by the specific architecture, the decreased metabolic activity or the presence of extracellular matrix (19). In addition, conditions in a processing environment such as temperature, have been shown to decrease biocide efficiency on biofilms (20, 21).

A major concern raised by the above observations is whether pathogenic microorganisms can be protected in mixed biofilms (22, 23). It has been demonstrated that *Bacillus subtilis*, resistant to peracetic acid exposure, was able to protect *Staphylococcus aureus*, usually sensitive to this disinfectant, in dual-species biofilm (24). Indeed, biofilms in food processing environments have been shown to contribute to foodborne pathogen survival in cleaning and disinfection treatments, leading to persistence of those microorganisms (25).

*Listeria monocytogenes* is a ubiquitous microorganism and it can cause serious foodborne disease. This psychotrophic bacterium has been found in different food products that can lead to listeriosis after ingestion (26). *L. monocytogenes* attaches to surfaces but its ability to form biofilms is controversial (27, 28). Nevertheless, clones of *L. monocytogenes* can survive and persist in niches of processing environments for several years despite cleaning and disinfection procedures (29, 30).

*S. aureus* is one of the most common causative agents of food-poisoning and is also involved in nosocomial infections (31). *S. aureus* can form biofilms on different abiotic surfaces found in food processing environment such as glass, stainless steel, polypropylene and polystyrene (32). The food processing environment can also provide suitable conditions for *S. aureus* biofilm production that is enhanced by sub-optimal growth temperature as well as glucose and sodium chloride availability (33). Furthermore, increase of resistance of *S. aureus* in biofilms towards disinfectants in processing environments has been reported in several studies (21).

Both pathogens are commonly found in food processing environment including dairy, meat and seafood worldwide (34, 35), and are reported to be common contaminating agents in the Brazilian dairy industry (36–38). Furthermore, several studies have highlighted that the two pathogens are not only found as mono-biofilm but rather in polymicrobial communities (6, 23, 39, 40).

The effectiveness of biocides depends on the composition of the food soil, the temperature (3) as well as the antimicrobial used and the surface type (41), the treatment exposure time and the procedure used (10). However, also the biofilm mode of growth is important for effectiveness of biocides. Therefore, the control of biofilm is important for public health in clinical or industrial environments and there is a need for understanding the mechanisms involved in the enhanced pathogen resistance seen in mixed-species biofilm. To date, most studies have focused on mono-species or dual-species biofilms; however, no studies have investigated biocide efficiency using more complex biofilm communities.

The purpose of this study was to determine if a “reproducible” mixed-species biofilm model could be established by co-cultivating a pathogen with an associate microbial community isolated from the food

processing sector. Specifically, we sought to determine if foodborne pathogens, such as *S. aureus* and *L. monocytogenes*, could establish themselves in such a mixed community and how the presence of this more natural scenario affected their sensitivity to commonly used biocides.

## Results

**Identification of the community members.** We isolated *L. monocytogenes* and *S. aureus* from two separate samples in Brazilian dairies (42, 43), and subsequently from each of these samples, isolated four different microbial strains at random (Table 1). The sample containing *L. monocytogenes* BZ001 (42) also contained *Klebsiella* sp. (BZ002), *Escherichia coli* (BZ003), *Comamonas* sp. (BZ004) and *Acinetobacter* sp. (BZ006). The sample containing *S. aureus* BZ012 (43) also contained *Aeromonas* spp. (BZ013), *Lactococcus lactis* (BZ014), *Candida tropicalis* (BZ017) and *Lactobacillus* sp. (BZ018).

**Establishment of the pathogen in a dual-species biofilm.** *S. aureus* and *L. monocytogenes* were individually grown in dual-species biofilms with each of their community members (Fig.1). The total sessile cell counts ranged from  $3.5 \times 10^6$  to  $3.8 \times 10^7$  CFU/cm<sup>2</sup> while *L. monocytogenes* counts ranged between  $1.8 \times 10^5$  and  $1.3 \times 10^7$  CFU/cm<sup>2</sup> in the dual-species biofilms. The total sessile cell count for the *S. aureus* communities ranged from  $3.9 \times 10^6$  to  $9.2 \times 10^7$  CFU/cm<sup>2</sup> and was between  $1.0 \times 10^6$  and  $6.9 \times 10^7$  CFU/cm<sup>2</sup> for the pathogen in the dual-species biofilms.

**Stable concentration of the pathogen in mixed-five-species biofilm.** In the *S. aureus* mixed-five-species biofilm, the total sessile count was  $1.9 \times 10^6$  CFU/cm<sup>2</sup> and the *S. aureus* sessile cell count was  $4.5 \times 10^5$  CFU/cm<sup>2</sup> (Fig. 2). The two different *S. aureus* isolates, BZ012 or Sa30, behaved very similarly in the mixed biofilm (data not shown). The total sessile cell count of the *L. monocytogenes* community was  $1.8 \times 10^7$  CFU/cm<sup>2</sup> and  $2.3 \times 10^5$  CFU/cm<sup>2</sup> for *L. monocytogenes* sessile cells. All members of the five species

communities remained in the mature biofilm based on the recovery of all members' colony morphology on BHI plates and by PCR detection for the *S. aureus* community (data not shown).

**MIC of the biocides.** The MIC of peracetic acid for both pathogens was 0.075 % (1/4 of the concentration used in the dairy) when grown as mono-culture. MIC of peracetic acid against the five-species grown together was 0.075 % for the mixed-culture containing *L. monocytogenes*, and 0.015 % (1/20 of the concentration used in the dairy) for the mixed-culture containing *S. aureus*. The individual MIC of peracetic acid against the associated microorganisms were 0.075 % for the four associate microbial community members of comLm (BZ002, BZ003, BZ004, BZ006), 0.075 % for BZ0013, 0.15% for BZ014 and BZ017 and 0.3 % for BZ018.

The chlorhexidine digluconate MIC for *L. monocytogenes* was 0.000390625 % and 0.0001953125 % for *S. aureus*. MIC of chlorhexidine digluconate against the five-species of the *L. monocytogenes* associate microbial community grown together was 0.003125 %. The MIC for chlorhexidine digluconate was not determined for the five-species-culture containing *S. aureus* as it was above 0.025 % which was the maximum concentration that could be tested without precipitation.

**The full community composition influences the biocide susceptibility.** Using the mono- and mixed-five-species biofilm model described above, biocide susceptibility was assessed. The exposure of a mono-biofilm of *S. aureus* to increasing concentrations of peracetic acid led to a sequential decrease of the *S. aureus* sessile cell survival (Fig 3A) of 2.5 log between the exposure at 0 % and 0.0375 %, then a 2.1 log reduction between 0.0375 and 0.075 % (MIC value). When *S. aureus* was part of a mixed community biofilms, 0.075 % peracetic acid only caused a 1.1 log reduction of the *S. aureus* sessile cells. This is a significantly ( $p = 0.0002$ ) lower reduction than seen for the mono-biofilm (4.6 log). Increasing the peracetic acid concentration from 0.075 % to 0.15 % fully eradicated *S. aureus* sessile cells from an initial concentration of  $4.7 \times 10^6$  CFU/cm<sup>2</sup> in a mono-biofilm and  $5.5 \times 10^5$  CFU/cm<sup>2</sup> in a mixed-five-species



biofilm (Fig. 3A). At the same time, no associate microbial community member sessile cells were recovered when the mixed-five-species biofilm was exposed to 0.15 % peracetic acid (Fig. 3A), meaning that less than 10 CFU/cm<sup>2</sup> were on the SSC if not all eradicated.

Treatment of a *L. monocytogenes* mono-biofilm with 0.0375 % peracetic acid caused a 3 log reduction of the sessile cells (Fig. 3B). The same treatment of the mixed-five-species biofilm led to a 2 log reduction of *L. monocytogenes* sessile cells while the total sessile cells decreased by 3.7 log. When exposed to higher concentration of peracetic acid *e. g.* 0.075 %, no viable *L. monocytogenes* sessile cells were recovered from a mono-biofilm. The total sessile cells and the *L. monocytogenes* sessile cells decreased by 0.8 log and 0.3 log, respectively, in the mixed-five-species biofilm treated with 0.075 % peracetic acid compared to treatment with 0.0375 % (Fig. 3B). No viable sessile cells were recovered when *L. monocytogenes* was grown as a mono-species biofilm or in a mixed-five-species biofilm treated with 0.15 % peracetic acid (2 fold higher than the MIC) (Fig. 3B).

**No effect of chlorhexidine digluconate was observed.** In order to assess susceptibility to other biocides used in the processing environment, the effect of chlorhexidine digluconate was also evaluated (Fig. 4). No effect of chlorhexidine treatment was observed on *S. aureus* grown as mono-biofilm or as part of mixed-five-species biofilm (Fig. 4A).

There was no impact of chlorhexidine digluconate treatment on *L. monocytogenes* for concentrations below 0.0125 % (Fig. 4B) whenever the pathogen was grown as a mono-biofilm or as part of a mixed-five-species biofilm. *L. monocytogenes* sessile cell counts remained steady in a mono-biofilm with 10<sup>4</sup> CFU/cm<sup>2</sup> when treated with concentration below 0.0125 % of chlorhexidine digluconate while it decreased from 1.6 x 10<sup>3</sup> CFU/cm<sup>2</sup> to 1.2 x 10<sup>2</sup> CFU/cm<sup>2</sup> in a mixed-five-species biofilm (Fig. 4B). This 1.2 log reduction was observed in the mono-biofilm when the sessile cells were exposed from 0.0125 % to 0.25 % chlorhexidine digluconate. The concentration of the total sessile cells, being of 4 x 10<sup>6</sup> CFU/cm<sup>2</sup>, did not change when

exposed to 0.00625 % and 0.0125 %, but decreased 1.3 log when exposed to 0.025 %. Concentrations higher than 0.025 % were not investigated due to technical limitations, as the chlorhexidine digluconate precipitated.

**The community rather than the individual influences peracetic acid susceptibility.** To determine if the altered sensitivity to peracetic acid in a mixed species biofilm was due to the associate microbial community or to the pathogen, we interchanged the pathogens and the communities. When *S. aureus* was grown with the *L. monocytogenes* community members, on the SSC not treated with peracetic acid, the total sessile cells count was  $1.3 \times 10^8$  CFU/cm<sup>2</sup> and *S. aureus* sessile cells count was  $2.5 \times 10^4$  CFU/cm<sup>2</sup> (Fig. 5A). No cells were recovered after treatment with 0.075% peracetic acid indicating that the total sessile cells decreased of at least 6.3 log ( $p = 0.001$ ) and the pathogen sessile cells decreased of at least 3.1 log ( $p < 0.001$ ). When *L. monocytogenes* was grown with the *S. aureus* community members, the overall biofilm production on SSC did not change (Fig. 3B and 5B). Treatment with 0.075 % peracetic acid led to a reduction of the total sessile cells by 3.4 log ( $p < 0.001$ ) and to 1.9 log of the *L. monocytogenes* sessile cells (Fig. 5B).

## Discussion

In environments, either natural, clinical or industrial, microorganisms mainly live in biofilms that are well structured communities and mostly composed of more than one microbial species (5, 6). However, in natural or man-made environment, the quantity and diversity of present species raise the complexity underlying the behavior of the biofilms compared to their single species growth behavior (7). Pathogenic microorganisms can also form biofilm or inhabit biofilms and this can cause problems for human health, especially if biocide treatment is not fully effective. Also, survival of microorganisms after biocide exposure is one of the results of the behavior modification. To date, most biofilm studies focusing on their mechanistic properties or antimicrobial resistance have been done on mono-species biofilms which do not reflect the complexity reached in mixed-community. Recently, some studies on mixed-biofilms have also been conducted but they have been limited on species diversity (44–47) without focusing on a specific pathogenic organisms.

In this study, we set up and studied two mixed-five-species biofilms containing a pathogenic bacterium. The ability of some strains to form biofilm fluctuated with some dual-species showing better ability to form biofilm than others depending on the strain association. Norwood *et al.* (48) have shown that *Pseudomonas fragi* and *S. xylosus* were the predominant species in biofilm with *L. monocytogenes*, and they described, as have other studies, that some microorganisms can take over in mixed-species biofilms. However, no strains were inhibited in our study. Both in the dual-species and the mixed-five-species biofilms, all strains remained in the mature biofilm, including both pathogens. A previous study on mixed-biofilm composed of *L. monocytogenes* and *Enterococcus* spp. found that growth at 25°C on stainless steel gave the highest biofilm cell counts (44), supporting the temperature selection that was used in this present study. Furthermore, the mixed-five-species biofilm model here was stable and reproducible; leading to a biofilm tool which can be used to study the behavior of pathogen in mixed-biofilms and to unravel mechanisms involved in survival or persistence towards antimicrobial compounds such as biocides.

*L. monocytogenes* is commonly described as non-/weak mono-layered biofilm producer (23, 49) but can colonize and persist in mixed-species biofilms (23) as also found in the present study. Some authors have

described that *L. monocytogenes* was inhibited in biofilms composed of several bacteria as compared to mono-biofilm (40, 48, 50), *e. g.* *S. sciuri* prevented *L. monocytogenes* from adhering and being part of a biofilm (50). In contrast, the sessile cell counts of *L. monocytogenes* on stainless steel were similar in mono- and mixed-five-species biofilms in this study, which is in accordance with other studies (40, 46, 51).

*S. aureus*, the other major pathogen selected is a renowned biofilm former (52), also in multispecies biofilms with *P. aeruginosa* (53, 54), *B. subtilis* (55), *E. faecalis* (56) or *C. albicans* (53, 57). Some studies have reported that lactic acid bacteria such as *Lactococcus* spp. and *Lactobacillus* spp. (58, 59) can inhibit growth of this pathogen while that was not noticed in this study. However, the sessile cell counts of each of the associate microbial community member including the pathogen decreased in the mixed-five-species biofilms compared to the sessile cell counts in mono- or dual-species biofilms which could be due to competition for nutriment and antimicrobial compound production (59).

Here, dairy sample communities were composed of various microorganisms representing the diversity existing in natural and man-made environment. Several studies have demonstrated that processing environments are composed of a large diversity of microorganisms (60, 61) and Dzieciol *et al.* (60) have shown that microbial communities were distinct depending on the collecting point, as observed in this study. Hence, it could be suggested that the microbial diversity in processing environments leading to the biofilm diversity, could impair effective disinfection procedure as Simões *et al.* (47) also concluded.

To date, very few studies have investigated antimicrobial resistance of mixed-species biofilms (46, 48) including comparison with mono-species biofilm data. Using the mature mixed-five-species biofilms on stainless steel coupon set up in this study, the biofilm models were subjected to biocides similar to those used in the processing environment where the strains were sampled. The effectiveness of biocide treatment on the two pathogens depended on the biocide used as previously shown (8, 44, 62). Peracetic acid treatment impaired survival in mono- and mixed-five-species biofilms while no significant effect of chlorhexidine digluconate was seen at the tested concentrations. Da Silva Fernandes *et al.* (44) have described peracetic acid as the most efficient biocide used in processing environment compared to quaternary ammonium,

sodium hypochlorite or biguanide and it was able to eliminate *L. monocytogenes* from the multi-species biofilm as observed in this study at a concentration of 0.15 %. Our results indicate that peracetic acid as used in the dairies (0.3 %) is efficient in reducing bacterial numbers since less than 10 CFU/cm<sup>2</sup> of sessile cells were recovered after treatment with 0.15 % peracetic acid (1/2 of the factory in-use concentration). The effect of chlorhexidine digluconate cannot be evaluated since the biocide precipitated at concentrations above 0.025 %.

Noteworthy, sessile cells of *S. aureus* were recovered from both mono- and mixed-five-species biofilm (comSa), when treated with 0.075 % peracetic acid. Also, *S. aureus* sessile cells survived in a mono-species biofilm at this concentration which was the MIC determined for this pathogen. This corroborates that biofilms can modify and increase biocide susceptibility response as suggested by some authors (8).

The mixed associate microbial communities, comSa or comLm, protected the pathogens during peracetic acid treatment. Thus, a 1 log difference was obtained between sessile cell counts of *L. monocytogenes* in mono- and mixed-five-species biofilms after biocide treatment. Using the same biocide, Van der Veen and Abee (63) also observed that *L. monocytogenes* was more resistant to peracetic acid in mixed-biofilm with *L. plantarum* than in mono-species biofilm. In the case of *S. aureus* sessile cells, a decreased of 2.1 log was obtained between treatment at 0.0375 % and 0.075 % peracetic acid of the mono-species biofilm while in a mixed-five-species biofilm the decrease was only of 0.2 log. Hence, similarly to Bridier *et al.*(55), *S. aureus* was less susceptible to peracetic acid exposure when the pathogen was part of a mixed-biofilm compared to growth as a mono-species biofilm.

We investigated if the increase of pathogen survival in mixed-species biofilm was due to specific ability of this pathogen in the associate microbial community or to the community itself. We interchanged the pathogens and the associate microbial communities *i.e.* *S. aureus* with comLm and *L. monocytogenes* with comSa. The new mixed-five-species biofilms were only treated with 0.075 % peracetic acid which was the highest concentration that allowed survival sessile cells recovery in the first set of experiments. *S. aureus* was established at a lower density in comLm than in the original comSa while the total sessile cell counts

262 increased. However, *S. aureus* was not protected by the comLm community and no *S. aureus* sessile cells  
263 were recovered. In the biofilm composed of *L. monocytogenes* with comSa and treated with 0.075% peracetic  
264 acid, a slight increase of survival sessile cells of *L. monocytogenes* was recovered compared to the  
265 association with comLm. This could be due to microbial composition variation, comLm being only  
266 composed of Gram negative bacteria while comSa was composed of Gram positive and negative bacteria as  
267 well as one yeast. Therefore, it is likely the full associate microbial community which is involved in the  
268 survival/resistance mechanisms. This protective effect could be due to the microbial species composition  
269 which influences the biofilm matrix composition as well as the presence of specific microorganisms with  
270 higher resistance to the tested biocide *e.g.* the MIC for peracetic acid of each member of comLm was 0.075%  
271 whereas members of comSa showed different MIC value ranging from 0.075 % to 0.3 % (concentration used  
272 in dairy). This is in agreement with the suggestion (64) that biocide resistance could be due to the  
273 extracellular polymeric substance of the matrix or to the environment but not only on the species specific  
274 attributes (46).

275  
276 In conclusion, biofilms can vary due to the microbial diversity encountered in man-made environments.  
277 This diversity is a key challenge to eradicate unwanted microorganisms. Further studies are required to  
278 unravel the exact mechanisms leading to the protective role of the community using complex microbial  
279 biofilm mimicking biofilms encountered in industry/clinical or natural environment as we attempted to set up  
280 in this study. Therefore, by improving the knowledge on mixed-species biofilm behavior in the presence of  
281 biocides, control of biofilm could be improving in any kind of sector.

## Materials and Methods

**Bacterial strains and growth media and biocides.** The strains of *L. monocytogenes* and *S. aureus* used in this study have been isolated from Brazilian dairies (42, 43) (Table 1). Four of the associate microbial community were also isolated as described below. They were isolated from the same sample as the pathogenic bacterium to obtain two communities as encountered in the dairy: one containing *L. monocytogenes* and one containing *S. aureus* (Table 1).

Two isolates of *S. aureus*, BZ012 and Sa30, were used in this study. The isolate BZ012 was selected representing the sequence type ST398 (43), a major possible health risk ST, and was used for dual-biofilm and mixed-species biofilm setup experiments. The isolate Sa30 was chosen as it represented the major ST/CC trend, ST30/CC1, found in the Brazilian dairy industry (43), and this isolate was used for assessing biocide effect on biofilm containing *S. aureus* (including the MIC assay). It was rationalized that the associate microbial community members isolated from the sample containing *S. aureus* BZ012 would be suitable for any *S. aureus* dairy isolates and therefore they were also used as community members with the *S. aureus* Sa30 isolate in the biocide susceptibility experiments.

Isolates were grown on Brain-Heart Infusion (BHI) broth (Oxoid), BHI agar (BHI broth, 1.5% agar, AppliChem) or Tryptic Soy Agar (TSA, Oxoid), Man Rogosa and Sharpe agar (MRS) and Dichloran Rose Bengal Chloramphenicol agar (DRBC, Oxoid, UK) and Potato Dextrose Agar plus chloramphenicol (PDA-CAM, Oxoid, UK). *S. aureus* was enumerated on Baird Parker agar (Oxoid, UK) with egg yolk emulsion (Oxoid, UK) and *L. monocytogenes* was counted on Oxford (Oxoid, UK) with modified *Listeria* selective supplement (Oxoid, UK). Unless otherwise specified, isolates were grown at 37°C and liquid cultures were incubated under shaking conditions at 250 rpm. The isolates were stored in BHI containing 20 % (vol/vol) glycerol (Merck, Germany) at -80°C. For biocide treatments, two biocides used in the dairies from where samples were taken were used: peracetic acid (Sigma-Aldrich, Germany) and chlorhexidine digluconate (Sigma-Aldrich, Germany). These stock solutions were diluted in NaCl 0.9 % (w/v) to the selected concentrations.



### Selection and identification of four associated microorganisms for each pathogen

**Selection of 4 associated microorganisms for each pathogen by phenotypical tests.** Using the same samples where the *L. monocytogenes* BZ001 (42) and *S. aureus* BZ012 (43) were isolated, four associate community members were isolated by surface plating 0.1 mL of a tenfold diluted sample in peptone water 0.1% supplemented with NaCl 0.85% (Oxoid, UK) suspension on BHI agar, MRS and DRBC. MRS and BHI agar plates were incubated at 30°C for 24 to 48 hours. DRBC plates were incubated at 25°C up to 7 days and then purified on PDA-CAM. Up to three different colonies of different morphologies were selected and purified on the respective culture media for subsequent analysis. Only one community *e.g.* four associate community members, for each pathogen species was selected and used in this study.

**Species identification.** The isolates were identified by phenotypic characterization and 16S rRNA or 28S rDNA gene sequencing. Shape and motility were determined by microscopy using an Olympus microscope (BX51). Gram-reaction was assessed by the 3% KOH method (65). Catalase or cytochrome oxidase was tested using 3 % hydrogen peroxide (Merck, Germany) and dry slide (BD Diagnostics, NJ, USA), respectively. DNA manipulation and 16S rRNA gene sequencing using the primer couple 27F (66) and 1492R (67) or NL1 (68) and LS2 (69) for the 28S rDNA were performed as described in Oxaran *et al.* (42) (Table 2).

**Development of mature biofilms on stainless steel coupon.** In order to reproduce the dairy processing environment, biofilm formation was assessed on stainless steel coupons (SSC) at 25°C (temperature noticed in the investigated dairies) using an associate microbial community of strains isolated from Brazilian dairies (42, 43). Overnight cultures were inoculated to a final OD<sub>600nm</sub> of 0.01 in a 5 mL BHI broth tube containing a 1 x 2 cm SSC (AISI 316, unpolished, 2B finish, prepared according to Kastbjerg and Gram (70)). Mono-species biofilms were produced from only one single strain, dual-species biofilms were composed of the pathogenic strain (*L. monocytogenes* or *S. aureus*) and one of the associate microbial community members, and mixed-five-species biofilms were obtained by the association of a pathogen strain and the four associate microbial community members listed in Table 1 (each inoculated at an equal ratio). Adhesion was performed



for 1 h 30 min at 25°C, with shaking at 90 rpm. Subsequently, the media was gently discarded to remove planktonic and loosely attached cells and replaced with BHI broth followed by incubation at 25°C, with shaking at 90 rpm. To obtain a mature biofilm, the *S. aureus*-containing biofilm was incubated for 25 h and the *L. monocytogenes*-containing biofilm for 72 h.

**Enumeration of planktonic and sessile cells.** Viable sessile cells in the mature biofilm on SSC were enumerated by determination of colony forming units (CFU). The SSC was gently washed three times with 2 mL NaCl 0.9 % and subsequently sonicated in 2 mL NaCl 0.9 % for 2 min (DELTA 220T, Aerosec Industrie). The CFU count of viable sessile cells was assessed by the drop plating method on BHI agar for the total cell count (including all the associate microbial community members), and for the model pathogen cell count, selective media was used *i. e.* BP or Oxford for *S. aureus* and *L. monocytogenes*, respectively. As growth control, the planktonic cell counts were done using the same method. The results considered the detection limit of the method used in this study which was 10 CFU/cm<sup>2</sup>.

**Species presence assessment.** To check that all associate microbial community members remained in the mature biofilm, two methods were used. For the community containing *L. monocytogenes*, species presence was evaluated by colony morphology on BHI agar, as the five community members showed distinct colony morphology. For the community containing *S. aureus*, colonies were not distinguishable on agar plate and PCR reaction using species specific primers (Table 2) was used to detect the different organisms. Genomic DNA (gDNA) was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) from the sessile cells using the same suspension used for enumeration, following the manufacturer's recommendations. PCR reactions were performed using the TEMPase Hot Start 2x Master Mix Blue II (Ampliqon, Denmark) according to the manufacturer's protocol. One µL of gDNA was added as matrix. The PCR reaction was run in a Veriti Thermal Cycler (Applied Biosystems, 96 Well Model 9902) and amplicon presence was checked by gel electrophoresis.

355 **MIC of biocides.** A first assessment of biocide susceptibility was performed using Minimum Inhibitory  
356 Concentration (MIC) assays in order to target concentrations to be used in the biofilm model. The  
357 concentrations used in the dairies were 0.3 % and 0.04 % for peracetic acid and chlorhexidine digluconate,  
358 respectively (S. H. I. Lee, personal communication). Each pathogen (*L. monocytogenes* and *S. aureus* Sa30)  
359 was assessed by MIC assay as mono-culture and mixed-culture (with the four associated microorganisms).  
360 The MIC assay was also done for the individual community members following the same protocol. Overnight  
361 cultures were adjusted to  $OD_{600nm} = 0.02$  in BHI and 100  $\mu$ l were dispensed into wells (96-well, round-  
362 bottom microtiter plate, Thermo Scientific, MA, USA) containing 100  $\mu$ l of a serial dilution of each biocide.  
363 Final biocide concentrations tested started at 4.8 % and decremented by 2 up to 13 times for peracetic acid  
364 and at 0.05 % and decremented by 2 up to 9 times for chlorhexidine digluconate. The MIC was determined  
365 after incubation for 24 h at 25°C, shaking at 90 rpm and was determined as the minimum concentration  
366 inhibiting growth.

367 **Biocide treatment of mature biofilm on stainless steel coupons.** After development of mature mono- or  
368 mixed-five-species biofilms, sessile cells were subjected to disinfection treatment. SSC were washed two  
369 times in 2 mL NaCl 0.9 % containing plates (6-well plate, Nunc, Denmark) and then immersed in a 2 mL  
370 NaCl 0.9 % solution at the selected biocide concentrations. Biocide treatment was stopped after 20 min of  
371 exposure (70) at 25°C, shaking at 90 rpm by transferring the SSC to 2 mL of Dey-Engley neutralizing broth.  
372 Then, the SSC was sonicated for 2 min in the neutralizing solution and enumeration was performed as  
373 described above.

374 **Statistic data analysis.** Statistical analyses were performed using the Excel Data Tool to process all data  
375 by variance analysis (ANOVA). Significance was defined with a Fisher test value as a  $P$  value  $\leq 0.05$ .

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- 569
- 570

**Figure legends****FIGURE 1**

Quantification of viable sessile cells on dual-species biofilms. Each pathogen, (A) *L. monocytogenes* (BZ001) or (B) *S. aureus* (BZ012) were grown as mono- or dual-biofilm developed on SSC with each of the associated community members. At least three biological replicates for *L. monocytogenes* and only duplicates for *S. aureus* were done.

**FIGURE 2**

Sessile cell counts of mono- and mixed-five-species mature biofilms. Pathogens were *L. monocytogenes* (BZ001) or *S. aureus* (Sa30). The mature biofilm was a 25 h biofilm and a 72 h biofilm for the *S. aureus* community and the *L. monocytogenes* community, respectively. Pathogen cell count was assessed on Baird Parker agar for *S. aureus* and Oxford agar for *L. monocytogenes*. Total community cell count was assessed on BHI agar. Error bars represent the standard deviation of three biological replicates.

**FIGURE 3**

Survival of sessile cells in biofilm formed on SSC after peracetic acid exposure. For each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes* (BZ001), mono- and mixed-five-species biofilms were developed on SSC as mature biofilm before being treated with different concentrations of peracetic acid (PA) for 20 min. Sa30: mono-biofilm of *S. aureus*; Sa30-comSa: mixed-five-species biofilms containing Sa30; BZ001: mono-biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are presented as the mean of biological triplicates.

**FIGURE 4**

Survival of sessile cells in biofilm formed on SSC after treatment with different concentrations of

595 chlorhexidine digluconate for 20 min. For each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes*  
596 (BZ001), mono- and mixed-five-species biofilms were developed on SSC as mature biofilm before treatment.  
597 Sa30: mono-biofilm of *S. aureus*; Sa30-comSa: mixed-five-species biofilms containing Sa30; BZ001: mono-  
598 biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are  
599 presented as the mean of biological triplicates for *L. monocytogenes* (B) and only one biological replicate for  
600 *S. aureus* (A).

601

## 602 **FIGURE 5**

603 Survival of sessile cells in mixed-species biofilm after 20 min with 0.075% peracetic acid (PA) treatment.  
604 Each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes* (BZ001) were grown as mixed-five-species  
605 biofilm developed on SSC with the other pathogen community members, *i.e.* BZ001 associated with comSa  
606 and Sa30 associated with comLm. Sa30-comLm: mixed-five-species biofilm containing Sa30; BZ001-com:  
607 mixed-five-species biofilm containing BZ001. Results are presented as the mean of biological triplicates.  
608

1 **TABLE 1** Bacterial strains used in the present study.

Pathogen / Community	Background microorganisms	Name	Reference
<i>L. monocytogenes</i>		BZ001	(52)
comLm	<i>Klebsiella</i> spp.	BZ002	This study
	<i>E. coli</i>	BZ003	This study
	<i>Comamonas</i> spp.	BZ004	This study
	<i>Acinetobacter</i> spp.	BZ006	This study
<i>S. aureus</i>		BZ012	(53)
		Sa30	(53)
comSa	<i>Aeromonas</i> sp.	BZ013	This study
	<i>Lactococcus lactis</i>	BZ014	This study
	<i>Candida tropicalis</i>	BZ017	This study
	<i>Lactobacillus</i> sp.	BZ018	This study

2

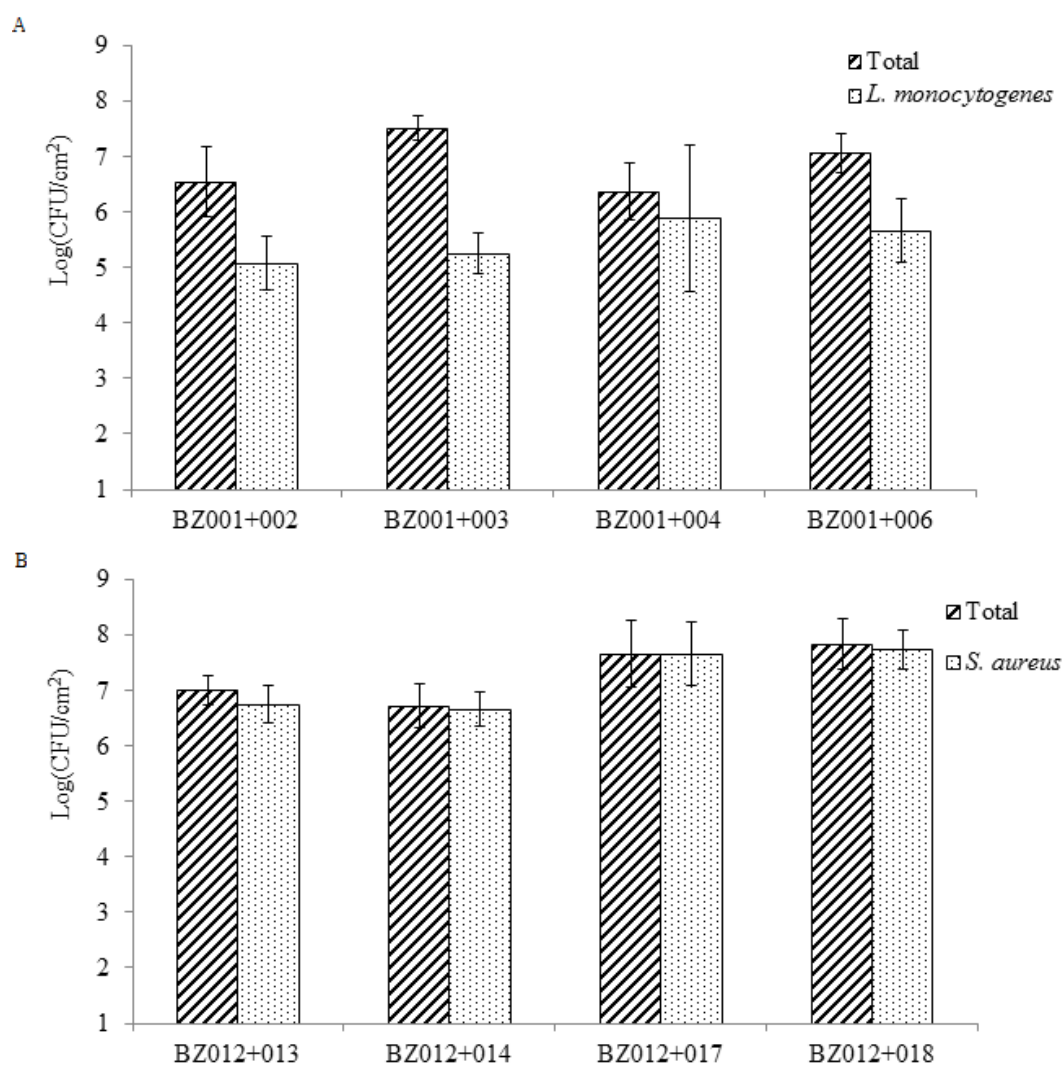
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4 **TABLE 2** Primers for species identification.

Species	Primer	Sequence (5'-3')	Reference
	27F	AGAGTTTGATCCTGGCTCAG	(87)
	1492R	GGTTACCTTGTTACGACTT	(88)
	NL1	GCCATATCAATAAGCGGAGGAAAAG	(89)
	LS2	ATTCCCAAACAACCTCGACTC	(90)
<i>S. aureus</i>	16S-Saureus-F	ATACAAAGGGCAGCGAAACC	This study
	16S-Saureus-R	TCATTTGTCCACCTTCGAC	This study
<i>L. lactis</i>	16S-Llactis-F	AAGTTGAGCGCTGAAGGTTG	This study
	16S-Llactis-R	AACGCGGGATCATCTTTG	This study
<i>Lactobacillus</i> spp.	16S-Lbacillus-F	ATTTTGGTCCGCAACGAG	This study
	16S-Lbacillus-R	ACCCACCAACAAGCTAATG	This study
<i>Aeromonas</i> spp.	16S-Aerom-F	GCAGCGGGAAAGTAGCTTG	This study
	16S-Aerom-R	ATATCCAATCGCGCAAGG	This study
<i>C. tropicalis</i>	16S-Ctropicalis-F	GTCGAGTTGTTTGGGAATGC	This study
	16S-Ctropicalis-R	ATCTCAAGCCCTTCCCTTTC	This study

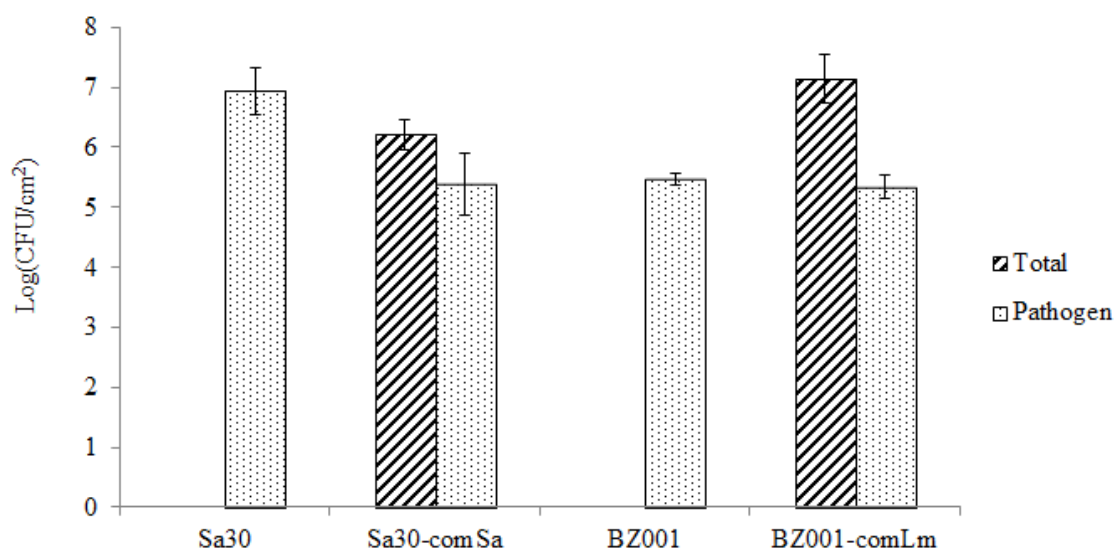
5

6



**FIGURE 1**

Quantification of viable sessile cells on dual-species biofilms. Each pathogen, (A) *L. monocytogenes* (BZ001) or (B) *S. aureus* (BZ012) were grown as mono- or dual-biofilm developed on SSC with each of the associated community members. At least three biological replicates for *L. monocytogenes* and only duplicates for *S. aureus* were done.

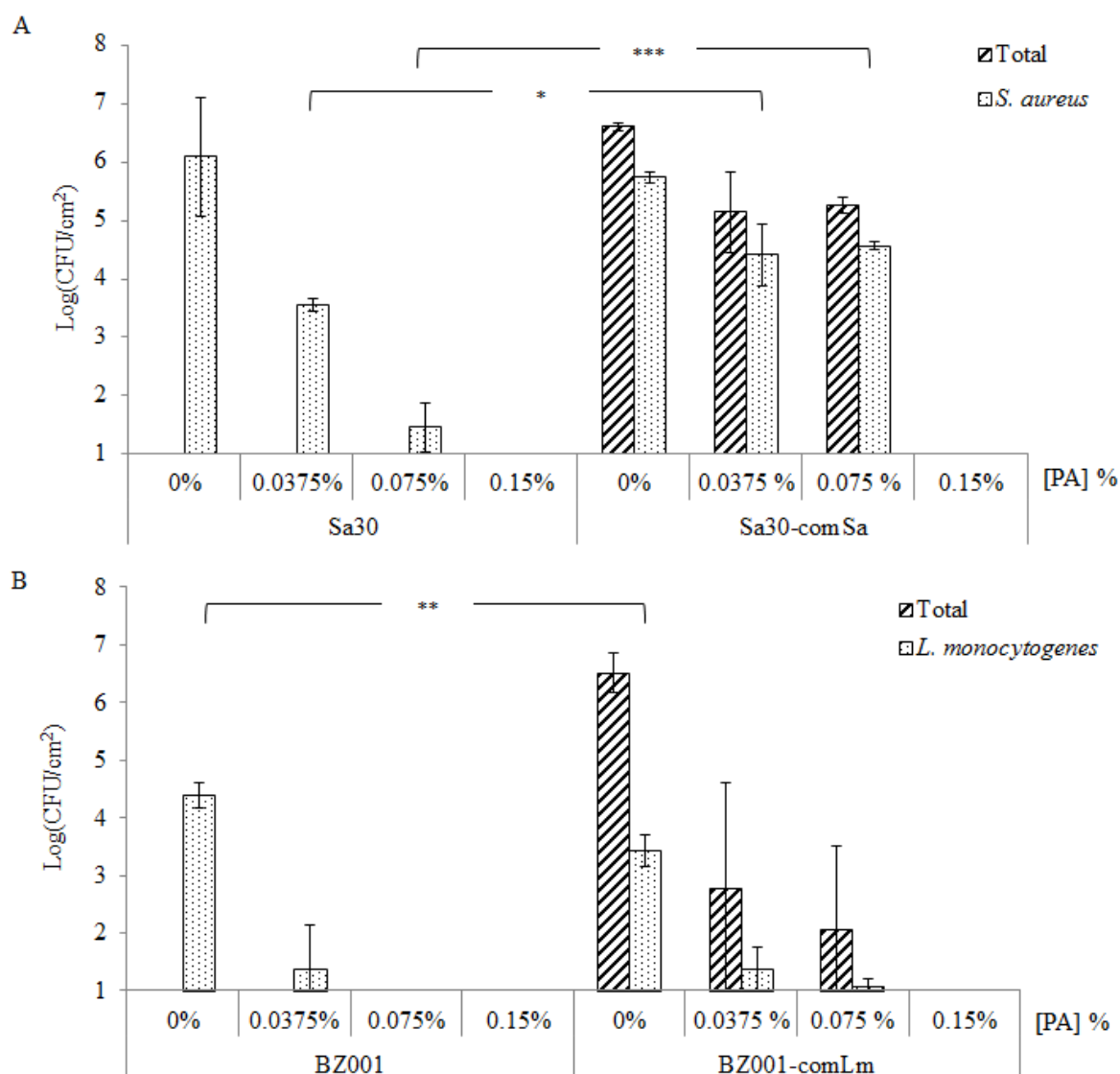


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**FIGURE 2**

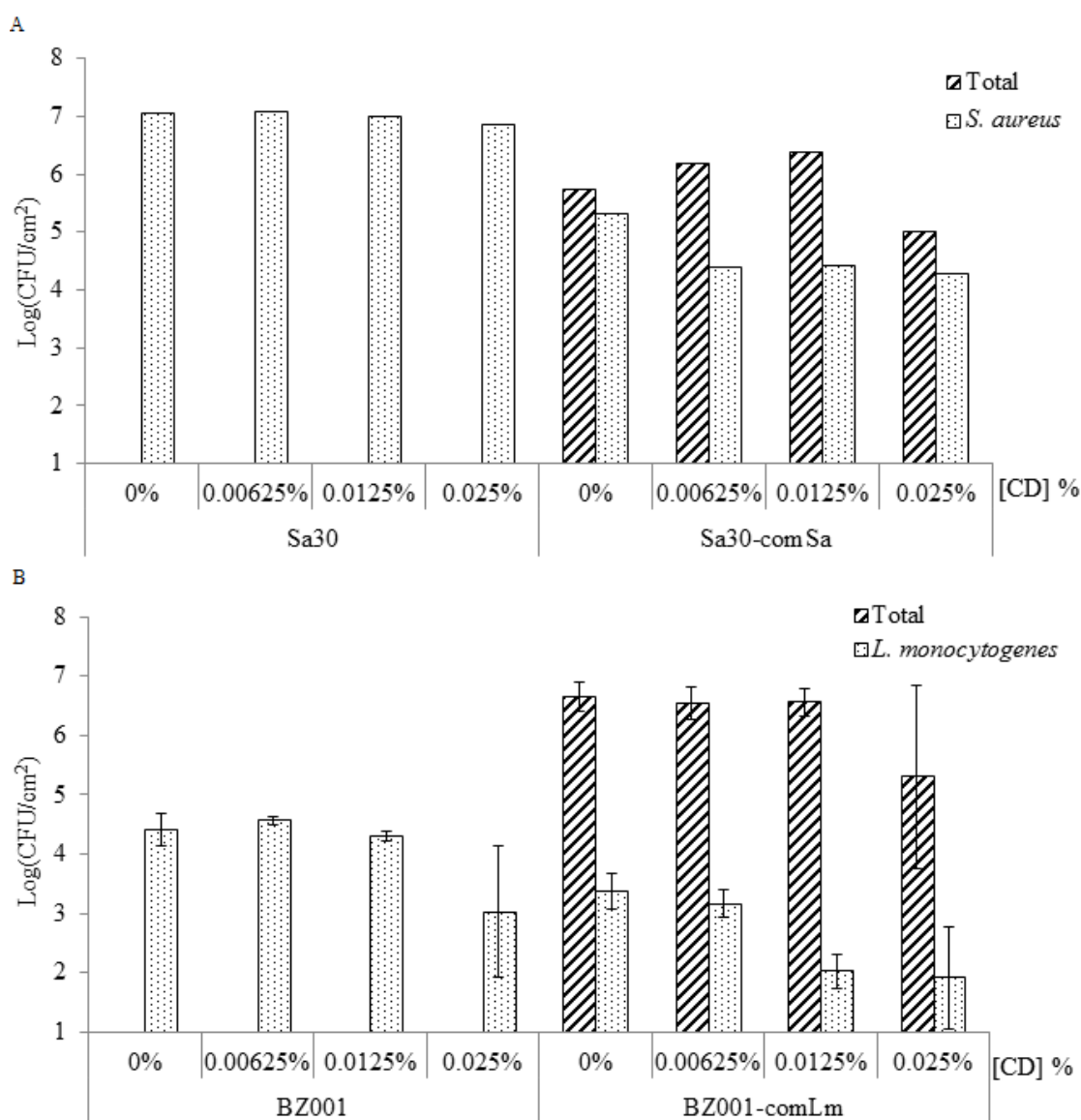
Sessile cell counts of mono- and mixed-five-species mature biofilms. Pathogens were *L. monocytogenes* (BZ001) or *S. aureus* (Sa30). The mature biofilm was a 25 h biofilm and a 72 h biofilm for the *S. aureus* community and the *L. monocytogenes* community, respectively. Pathogen cell count was assessed on Baird Parker agar for *S. aureus* and Oxford agar for *L. monocytogenes*. Total community cell count was assessed on BHI agar. Error bars represent the standard deviation of three biological replicates.





**FIGURE 3**

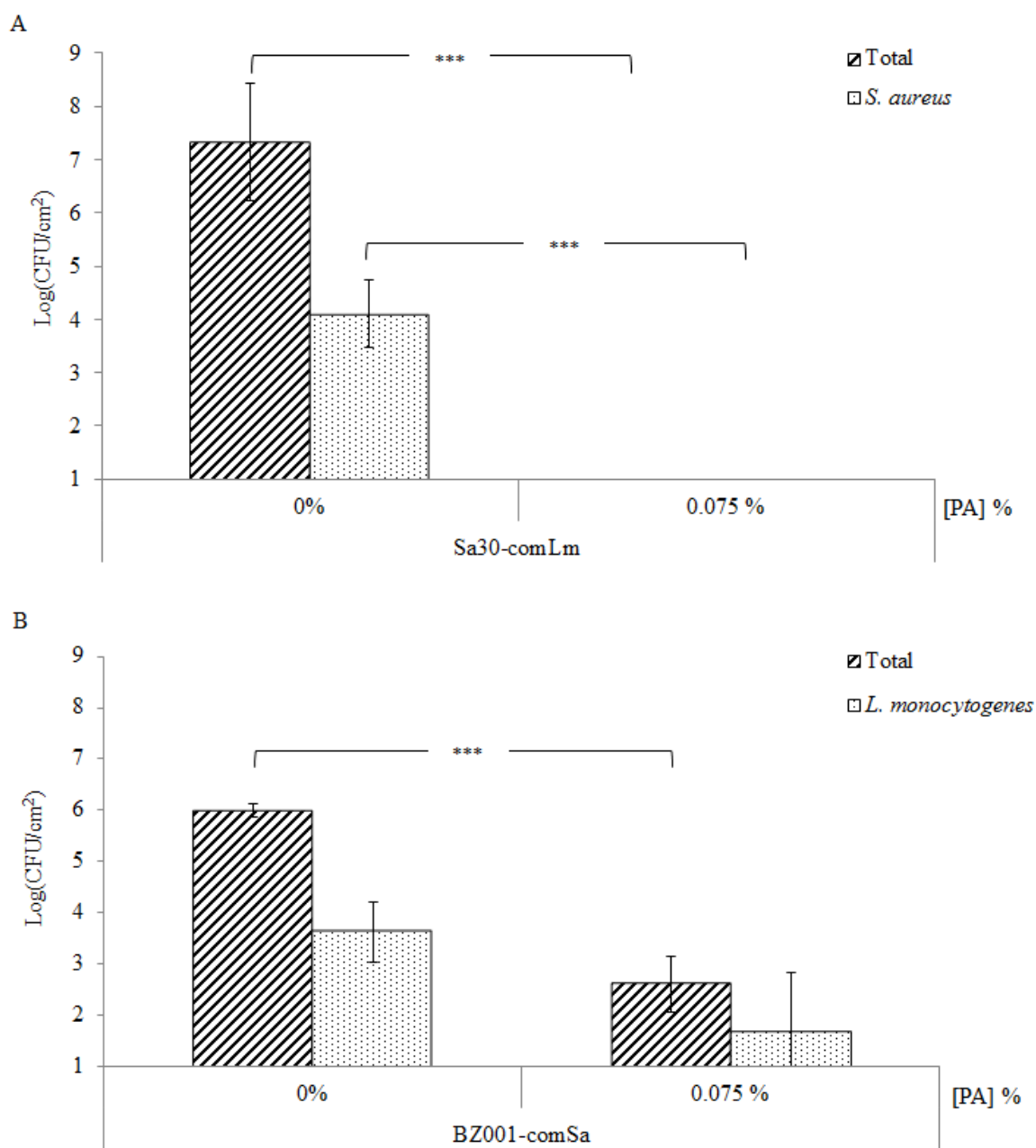
Survival of sessile cells in biofilm formed on SSC after peracetic acid exposure. For each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes* (BZ001), mono- and mixed-five-species biofilms were developed on SSC as mature biofilm before being treated with different concentrations of peracetic acid (PA) for 20 min. Sa30: mono-biofilm of *S. aureus*; Sa30-comSa: mixed-five-species biofilms containing Sa30; BZ001: mono-biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are presented as the mean of biological triplicates.



**FIGURE 4**

Survival of sessile cells in biofilm formed on SSC after treatment with different concentrations of chlorhexidine digluconate for 20 min. For each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes* (BZ001), mono- and mixed-five-species biofilms were developed on SSC as mature biofilm before treatment. Sa30: mono-biofilm of *S. aureus*; Sa30-comSa: mixed-five-species biofilms containing Sa30; BZ001: mono-

29 biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are  
30 presented as the mean



**FIGURE 5**

Survival of sessile cells in mixed-species biofilm after 20 min with 0.075% peracetic acid (PA) treatment.

Each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes* (BZ001) were grown as mixed-five-species

biofilm developed on SSC with the other pathogen community members, *i.e.* BZ001 associated with comSa

- 36 and Sa30 associated with comLm. Sa30-comLm: mixed-five-species biofilm containing Sa30; BZ001-com:  
37 mixed-five-species biofilm containing BZ001. Results are presented as the mean of biological triplicates.